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Express Mail Label No.

04/17/03

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Mary	Eaton	Miami, FL

☐ *Additional inventors are being named on the _____ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

Use of clonal human hNT2 neuronal cell lines as "cellular minipumps" to treat neurodegenerative disorders and the consequences of brain and spinal cord injuries.

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☑ Specification *Number of Pages* 9 ☐ CD(s), Number _____

☑ Drawing(s) *Number of Sheets* _____ ☐ Other (specify) _____

☐ Application Data Sheet. See 37 CFR 1.76

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☐ No.

☑ Yes, the name of the U.S. Government agency and the Government contract number are: Veterans Affairs

Respectfully submitted,

SIGNATURE Michael J. Gonet

Date 18 Apr 03

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REGISTRATION NO. *(if appropriate)* 31465

Docket Number: VA 02-106

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Date: 09/03/2002

REPORT OF INVENTION

1. Title of Invention: Use of clonal human hNT2 neuronal cell lines as 'cellular minipumps' to treat neurodegenerative disorders and the consequences of brain and spinal cord injuries.

2. Name/address of Inventor:
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3. Research duties for the VA: A Principal Investigator at the Miami VAMC, with a joint appointment with The Miami Project, at the University of Miami School of Medicine, who conducts research related to spinal cord injury and its sequella, with an emphasis on utilizing neural cell transplantation to improve the consequences of SCI. The invention was created intellectually while at The Miami Project.

a. position description: see attached
b. IPA: no IPA

4. Name/address of VA facility: Miami VA Medical Center
1201 NW 16th Street
Miami, Fl 33125

5. Contribution of the VA to the invention:
Beginning 7/01/2001, a project conducted by the PI, from a grant supported by RR&D VA partially supported the implementation of the invention. Since that time, the VA has provided 5/8 salary support for the inventor. Project funds equal to $44, 000 supported the purchase of tissue culture and surgery supplies, for animal purchase and their care, and for the purchase of behavioral testing equipment. To date, all experiments related to the invention have been performed at the PI's facilities at The Miami Project, at the University of Miami.

6. All other government contribution: none

7. Other non-government contribution:
The Miami Project to Cure Paralysis, University of Miami School of Medicine,

has provided facilities, equipment, technical staff support, including their salaries, the balance of the Inventor's salary, and considerable funds for tissue culture and other supplies needed for the implementation of the invention. An estimate of those funds would include: $50, 000 for supplies, and about $110,000 for the salaries of staff utilized and the PI, for the time period 7/1/01-8/15/02. Most of those University support funds were directly related to implementation of this invention. Other grant funds during that time period were used for other projects.

8. Invention description:

a. General Purpose–

Chronic pain and spasticity constitute some of the significant problems following spinal cord injuries (SCI), interfering with rehabilitation and daily activities, which in turn leads to significant complications in these patients. However, there are no effective, completely safe therapies for the reduction of these conditions (and other related to neural trauma and degeneration). As a means to ameliorate these problems, we propose to create novel human neural cell lines, subcloned and derived from the human NT2 cell line, where each subcloned cell line synthesizes and secretes bioactive agents such GABA, the opioid met enkephalin (MET-ENK), or serotonin (5HT). Transplants of these secreting human neural cell lines are proposed to reduce consequences of spinal trauma after transplant in or near the spinal cord.

b. Background (Prior Art)--

Oral baclofen, the GABA-B receptor agonist, has often been the drug of choice for spasticity [2] due to spinal cord trauma, and baclofen and opioids have been used for the relief of chronic pain [15]. However, oral baclofen is often ineffective at non-toxic doses, because large doses are required to cross the blood-brain barrier and leads to subsequent CNS effects [12], usually not well tolerated by spastic patients. Intrathecally administered baclofen is currently FDA-approved for those patients with spinal cord injury who are refractory or can not tolerate oral baclofen, but spinally-delivered baclofen via programmable implantable electronic pumps are also fraught with problems, such as high costs, catheter twisting, infection at the implant site, overdosing and the development of tolerance with increasing doses of baclofen required for relief [17,25]. Furthermore, the efficacy of baclofen pumps is mainly in terms of lower extremity spasticity and the treatment has little effect on upper extremity spasticity. Intrathecal morphine has been used via electronic pump and has been prescribed for use in neuropathic pain, but often has complications of tolerance, overdose, and problems related to the pump itself [13,19,23].

Transplants of primary cultured cells near the dorsal horn of the spinal cord that release peptides and neurotransmitters have offered a new direction in the treatment of chronic pain[22]. But, primary cells are difficult to obtain, non-homogeneous, and would require that each batch be tested before clinical use. Transplantation of immortalized cell lines genetically-modified to release neuroactive antinociceptive peptides [24], inhibitory neurotransmitters [7,9] and neurotrophins [4] in chronic pain, and to upregulate inhibitory neurotransmitter synthesis [11] offers a renewable source of cells that can act as cellular minipumps, are able to respond to the microenvironment of the cord, and should reduce or eliminate side effects associated with the large doses of pharmacologic agents required for centrally-acting pain-reducing agents. A naturally-immortalized human embryonal carcinoma cell line, NTera2cl.D/l (NT2), differentiates

irreversibly into several morphologically and phenotypically distinct cell types, which include terminally differentiated postmitotoc CNS neurons [1]. Successive re-plating of retinoic acid-treated NT2 cells, in the presence of growth inhibitors, results in the isolation of purified human neurons [16], that have been extensively characterized and tested in vivo in a number of animal models of traumatic injury and neurodegenerative disease [3]. The potential application of NT2 neurons in cell transplantation therapy for CNS disorders, and their use as vehicles for delivering exogenous proteins into the human brain for gene therapy, has been recently demonstrated [21]. Such NT2 neurons are being currently used in Phase II clinical trials for the treatment of stroke, and have been approved by FDA for such trials [14]. However, such NT2 cells contain a variety of neural phenotypes, and would provide a plethora of neuroactive substances if used for the specific treatment of problems such as pain or SCI-associated spasticity.

This invention proposes to isolate novel and specific, well-characterized cell lines from the hNT2 parent line by ordinary subcloning methods, and test the use of individual novel NT2 cell lines for the treatment of traumatic consequences, such as pain and spasticity. However, such cell line transplants could also be used to treat a variety of neural trauma and neurodegenerative problems, including epilepsy, depression, mobility and sensory disorders, including Parkinson's and Alzheimer's disease.

The basis of the proposed efficacy of this invention is that release of neuroactive agents from such cell transplants will provide a low, local dose of agent in the area of neural substrate where needed, without a broad systemic effect, and such cells, since immortalized and grown in vitro can be well-characterized and tested for homogeneity and safety for human use.

c. Description and Operation–

Subcloning and characterization of NT2 GABA, 5HT, and Met-ENK neuronal cell lines. We use standard methods for serial dilution and cloning rings to isolate proliferating and rapidly-growing colonies of the parental hNT2 cells, that form cell lines, in vitro [8]. We are using a more rapid cell-aggregation method [5], described below, for the differentiation of proliferating NT2 cells that only requires 14 days of retinoic acid (RA) treatment, to characterize each NT2 clonal line for a GABA phenotype. Each clonal line will be initially stained for GABA , 5HT, or Met-ENK production with immunohistochemical methods [9] using commercially-available antibodies directed against GABA, 5HT or MET-ENK. GABA-, 5HT-, or MET-ENK-containing NT2 cell lines will be double-labeled with the neuronal marker, human neuronal nuclear marker (NeuN) [18], or NuMA [6] to insure the phenotype is both GABAergic, 5HTergic or MET-ENKergic and neuronal. The content and secretion of the neurotransmitter GABA or 5HT from candidate GABA or 5HT NT2 cell lines will be evaluated by standard high performance liquid chromatography (HPLC) methods, described below, and have been used to characterize other neurotransmitter cell lines [9,10]. Cell lines which synthesize and release greater than 100pmole GABA or 5HT/10^6 cells/min will be used for further characterization and transplant experiments. Some of the NT2 neuronal cell lines which do not synthesize or secrete GABA, 5HT, or MET-ENK and do not stain positive for these agents by antibody methods, but survive well in vitro will be used as the negative-control NT2 cell line for further transplant work in models of pain and spasticity after SCI.

Rapid aggregation method for differentiation and identification of NT2 clones. This method was adapted from an earlier published aggregation method for NT2 cell cultures[5]. Confluent proliferating NT2 cells, expanded in non-tissue culture 100mm plates, are treated for two weeks with 10μM all-trans retinoic acid (RA), in high-glucose DMEM media, made pH 8.0 with 15mM HEPES buffer. Remaining

cells are replated in poly-L-lysine/laminin-(PLS/lam)coated tissue culture plates (20ug/ml), and the high-glucose DMEM supplemented with 2mM Glutamine, pH 7.4. Twenty-four hours after replating, cytosine-D-arabinofuranoside (araC), 1μM, and uridine, 10μM are added to the media for seven days. Differentiation is continued for two and four weeks after the removal of the mitogen inhibitors on PLS/lam-coated plastic slides. Individual clonal lines are examined for antibody staining and characterization for GABA, 5HT, or MET-ENK and NeuN and other markers.

Immunohistochemistry methods: in vitro. Staining for GABA, 5HT or MET-ENK in NT2 cell lines will proceed as published previously[9]. The specific GABA, 5HT or MET-ENK signal will be examined in both proliferating and differentiated cell lines. Any suspected GABA or 5HT cell lines will also be examined for GABA or 5HT content and release by HPLC (below)to insure that any suspected GABA or 5HT synthesis is authentic. Putative MET-ENK cell lines will be examined for MET-ENK release by examination of the cell-conditioned media for its ability to decrease cAMP content in CHO cells which express the μ-receptor for opioids [20].

Methods for NeuN staining have been previously published [18]. NeuN and NuMA will be examined in differentiated clonal cultures to insure that clonal cell lines are human neurons. Typical methods for in vitro GABA, NeuN, and NUMA staining are as follows. Fix cells with Lana's (4% paraformaldehyde plus 10% picric acid, pH 7.4) at 4 C for ten minutes. Rinse and permeabilize cells with 0.4% Triton X-100 in PBS. Incubate ten minutes at room temperature. Incubate with primary antibody for 12 hrs at 4°C. For GABA: Guinea pig antisera raised against GABA (Protos Biotech Corp.), 1:250 PBS. For NeuN: Mouse anti-neuronal Nuclei monoclonal antibody (Chemicon) 1mg/ml., 1:100 PBS. For NuMA: Monoclonal NuMA (Ab-2) (Oncogene) 100 ug/ml., 1:100 PBS. Rinse and incubate with secondary antibody for 2 hrs. Typical secondary antibodies include: For GABA: Alexa Fluor 488 Goat anti-guinea pig (Molecular Probes), 1:150 PBS. For NeuN and NuMA: Alexa fluor 488 Goat anti-mouse (Molecular Probes), 1:150 PBS.

HPLC GABA in NT2 cell lines. Either GABA content is measured in water-lysed cells, or GABA secretion is measured in cell-conditioned media after three days of undisturbed incubation under proliferation or differentiation conditions. The HPLC system consists of a solvent-delivery pump (Waters 510 Pump), an autosampler (Waters 717 plus Autosampler) and an electrochemical detector (ESA Coulochem II; Electrode: ESA Model 5011 Analytic Cell; Guard Cell: Model 5020). Elution is carried out at room temperature with a reversed-phase column (3 uM, C18, 80x4.6, HR-80, ESA) and a mobile phase of 0.1 M Sodium Acetate (pH=5)-acetonitrile (73:27, v/v) at a flow rate of 0.6 ml/min. To an OPA solution (2 mg of o-Phthaldialdehyde (OPA) in 0.2 ml Methanol), 0.8 ml of 0.1 M Borax Buffer, pH 10.4 and 5 μl of 2-mercaptoethanol (2MCE) are added. 4 min before the injection on the column, to prepare the OPA reagent. The OPA reagent and sample are mixed (1:4) and incubated at room temperature in the autosampler before injection. After injection, the GABA peak appearance time is about 5 min in 27% Ace Mobile Phase. Determination of 5HT synthesis will follow similar published methods [10].

TEST DATA:

Some current examples of these human cloned cell lines include:

Figure 1: A serotonergic NT2 cell line

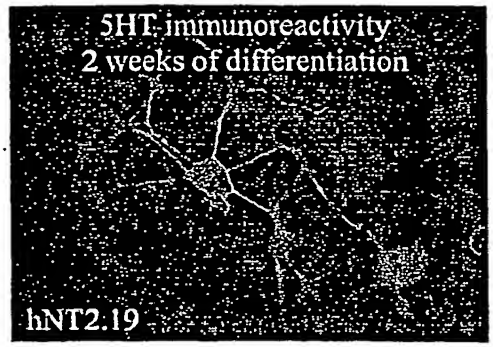

Fig.1. The hNT2.19 serotonergic cell line, subcloned by serial dilution and treated for two weeks with retinoic acid and mitotic inhibitors. They were further differentiated for two weeks before an antibody stain for serotonin (5HT). The 5HT NT2.19 cells, have a very large nucleus, are generally bipolar, with short neurites and stain brightly for 5HT.

Figure 2: Two GABAergic NT2 cell lines

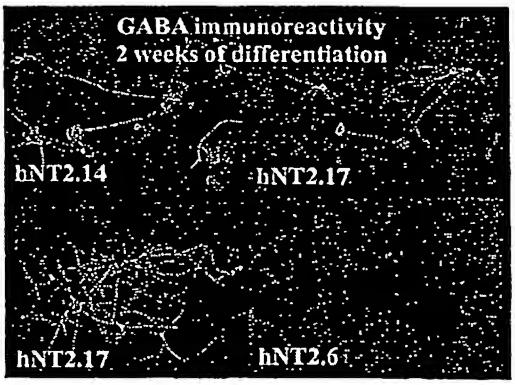

Fig. 2. Some other cloned hNT2 cell lines, two of which are positive for GABA (hNT2.14 and hNT2.17) are shown here. Each was subcloned by serial dilution and treated for two weeks with retinoic acid and mitotic inhibitors. They were further differentiated for two weeks before an antibody stain for GABA. The GABA NT2.17 and NT2.14 cell lines have small nuclei, extensive multipolar neurites and stain brightly for GABA. The NT2.6 cell line, does not stain either for GABA or 5HT, and will provide the negative control to the 5HT cell line transplants.

d. Non-technical description–

The availability of an inexhaustible supply of human cells which can make specific biologic agents to reverse the damage to the nervous system that could be safely used for transplant into or near the brain or spinal cord after injury or in disease states would be very useful to treat a variety of problems such as pain, spasticity, epilepsy, immobility or related problems. Ideally such cell sources should not be derived from human embryos or cadavers, and should not form tumors if placed in the patient, but would still function and survive for long periods. Such cell transplants would not be required to replace missing cells, but would function as cellular minipumps, releasing beneficial molecules near damaged tissue so that some of the damaged tissue and hence, human function, could be repaired or improved.

We have been able to derive some of these clinically useful cells by isolating unique colonies of daughter cells from a naturally-immortal human cell line called hNT2, so that the daughter colonies are different from each other and the original parent hNT2 cell type, a process called subcloning. However, these new daughter cell lines retain the property of being naturally-immortal, when they are grown in the absence of retinoic acid (RA). Is this condition, they multiply indefinitely, allowing them to be frozen, stored, and restarted at any time in culture. If the cells are treated with RA for a few weeks, they become human neurons, no longer capable of division. Such cells can be safely transplanted into the potential patient, with no danger of tumor formation, since they become human neurons irreversibly, which no longer divide and multiply. In addition, since they are unique derivative cell lines, they have characteristic features, such as the ability to make and secrete useful agents, such as the neurotransmitters serotonin (5HT) and gamma γaminobutyric acid (GABA).

We are testing the ability of transplants of these unique hNT2 cell lines to relieve chronic pain and spasticity in animal models after spinal cord injury (SCI), and expect them to be beneficial for these conditions. Transplant of cell lines that secrete 5HT or GABA that could be used in the human conditions, such as the consequences of SCI where chronic pain and spasticity are important problems clinically, would make them a desirable new tool for the SCI population. However, such subcloned cell lines, derived from the parental hNT2 neuronal cell line have great potential for cell therapy for the treatment of a variety of traumatic and neurodegenerative diseases in humans. Examples of what these cell could make include GABA, 5HT, catecholamines, opioids, many other peptides or agents unknown at this time.

9. Commercial possibilities:

Unique, well characterized human neuronal cell lines, which could safely be transplanted in or near the central or peripheral nervous system(PNS), such as peripheral nerves, the brain or spinal cord would be an improvement over currently used technologies, such as pharmacologic agents or implantable mechanical pumps. Drugs used for the treatment of these neurologic problems are often ineffective or resulting in deleterious side effects at an effective dose. Implantable mechanical pumps have been associated with many unacceptable problems such as, drug overdose, catheter kinking, pump failures, infection, and high costs. Cell line grafts would be done as a spinal tap, on an outpatient basis and could be re-injected as needed for continuing effectiveness. The cells are a potentially infinite source, frozen until needed, and can easily be tested at any time for any possible infectious agents or contaminants before use. If the technology is well developed the costs could be far lower than mechanical pumps or long term drug use.

10. Reasons for patent:

Both the conceptual idea as well as practical application of cell lines subcloned from hNT2 cells needs to be protected by patent, since the research & development required is of considerable expense. Eventually these cells lines will be used for clinical trials and need commercial development, which will require patent protection.

11. Other institutional interest:

The University of Miami and Office of Technology Transfer has tentatively expressed interest in pursuing a patent application for this invention.

12. Planned publications:

As soon as possible, perhaps as early as the Spring of 2003, I would like to present some of our data at the American Society for Neurotransplant and Repair as an abstract. A manuscript would soon follow in 2003.

13. Previous Disclosure:

This invention was previously disclosed to the University of Miami Office of Technology Transfer on 5/10/01, #UM-0057 with the title: "A transplantable human GABA neuronal cell line for the treatment of spasticity after spinal cord injury"

14. Patent Application #: N/A

15. Certification of VA Inventor: see attached

16. Execution--

Signature: Date: 9/03/01

Mary Eaton, PhD
Research Health Scientist
VAMC, Miami FL
and Asst. Prof. of Neurological
Surgery
University of Miami School of Medicine

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